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EVOLUTIONARY RELATIONSHIPS OF *POPENAIAS POPEII* AND THE EARLY EVOLUTION OF LAMPSILINE BIVALVES (UNIONIDAE): PHYLOGENETIC ANALYSES OF DNA AND AMINO ACID SEQUENCES FROM F AND M MITOCHONDRIAL GENOMES

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ABSTRACT

Lampsiline bivalves typically are considered a tribe within the Unionidae (Ambleminae: Lampsilini), and they display extraordinary morphological adaptations for reproduction. Recent studies have weakly corroborated the monophyly of the Lampsilini, but evolutionary relationships within the tribe and its sister lineage have yet to be elucidated convincingly. However, these determinations are necessary to better understand the evolution of the spectacular morphological diversity present in lampsilines, as well as the specific circumstances surrounding the group's origin. To clarify these matters, phylogenetic analyses were carried out on 2,310 nucleotide and 770 amino acid position matrices containing sequences from five protein coding gene regions on the F and M mitochondrial genomes from 21 amblemine species. Nodal support values on the best Bayesian inference tree robustly confirm the monophyly of lampsilines and a clade containing the following well-supported relationships: (((lampsilines, *Popenaias*) *Plectomerus*) *Amblema*). Furthermore, a maximum likelihood estimate of ancestral character states indicates that the ectobranchy observed in lampsilines + *Popenaias* is homologous and was derived from a tetragenous ancestral lineage. The sister taxon status of *P. popeii* to the traditional lampsiline taxa and the occasional use of the inner demibranchs for brooding suggest that this species could still retain character states of the lampsiline ancestral lineage. Therefore, additional studies of morphology, reproduction, phylogeography and ecology for *Popenaias*, *Amblema*, *Plectomerus*, and other taxa within the Amblemini could clarify the circumstances surrounding the origin of the lampsiline bivalves.

Key words: Cytochrome *c* oxidase subunits I & II (*cox1*, *cox2*), DUI, phylogenetics, Ambleminae, Unionidae, *Popenaias popeii*.

INTRODUCTION

Lampsiline bivalves (Unionidae: Ambleminae: Lampsilini; *sensu* Davis & Fuller, 1981) exhibit a considerable array of morphological and anatomical adaptations (Zanatta & Murphy, 2006). Taxa are endemic to eastern North and Central America, with 119 species in 21 genera currently recognized in the United States (Burch, 1975; Davis & Fuller, 1981; Turgeon et al., 1998; Roe & Hartfield, 2005). Generally regarded as a monophyletic group (Heard & Guckert, 1971; Davis & Fuller, 1981; Lydeard et al., 1996; Graf & Ó Foighil, 2000; Campbell et al., 2005; Zanatta & Murphy, 2006; but see Frierson, 1927), it was first recognized as such by Ihering (1901); however, diagnostic criteria were not specified until Ortmann (1910). Among these apparent diagnostic characteristics, the following features have been considered particularly definitive: (1) dorsal margin of inner lamina of inner gills generally entirely connected with abdominal sac (Ortmann, 1912); (2) mantle edge anteroventral to

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the incurrent, branchial opening in females crenulated, papillose, or with a structural elaboration, for example, a caruncle or flap whose motion is customarily interpreted as a lure to attract hosts (Ortmann, 1912; Heard & Guckert, 1971; Fuller, 1975; Davis & Fuller, 1981); (3) when gravid, marsupial water tubes extending below the ventral margin of the branchial filaments (Ortmann, 1912; Fuller, 1975; Davis & Fuller, 1981); and (4) larvae (glochidia) released from the mother through pores that develop at the ventral margin of the marsupial water-tubes (Ortmann, 1912). Ortmann, referring to the Lampsilinae, stated (1912: 301): "The latter physiological character [#4 above] is unique, and is found only in this subfamily." However, these "diagnostic" morphological characteristics likely do not represent unique and unreversed character states for lampsilines but rather generalities noted by Ortmann (1910, 1912) (e.g., glochidia are not released from the ventral margin of the marsupium in *Cyprogenia*, *Hamiota*, and *Dromus* – Chamberlain, 1934; Haag et al., 1995; Jones et al., 2004). Therefore, the answer to the question, "What is a lampsiline?" is potentially contentious. Evolutionary relationships among the nominal lampsiline genera are still poorly understood. *Cyprogenia*, *Cyrtonaias*, *Dromus*, *Friersonia*, *Obliquaria*, and *Ptychobranchus* usually are acknowledged as being relatively primitive members of the Lampsilini based on the marsupial morphology and relative lack of conchological sexual dimorphism (Ortmann, 1912; Heard & Guckert, 1971; Fuller, 1975; Davis & Fuller, 1981). The remaining genera ("heterogenae" [i.e., posteriorly located marsupia] of Simpson, 1900, 1914; Heard & Guckert, 1971; Davis & Fuller, 1981) are regarded as a relatively derived monophyletic group. Rather than relying on formal phylogenetic analyses, most assessments regarding supra- and inter-generic phylogenetic relationships within the Lampsilini were based on relatively small numbers of characteristics and the assumption that relatively simple structures are ancestral to relatively complex, specialized structures. Recent studies of lampsiline evolutionary relationships (Campbell et al., 2005; Zanatta & Murphy, 2006) have not robustly corroborated the monophyly of the taxon nor have they convincingly elucidated the relationships within the group or its sister lineage. These determinations are necessary to better understand the specific circumstances surrounding the origin of the Lampsilini and the evolution of its extraordinary morphological diversity.

Campbell et al. (2005) and Zanatta & Murphy (2006) currently represent the most comprehensive studies of lampsiline bivalve phylogeny. Their results associate *Popenaias popeii* (Lea, 1857) either (1) with a clade containing *Amblema* (Campbell et al., 2005: figs. 1, 2; Zanatta & Murphy, 2006: fig. 1) or (2) as a potential sister taxon to the Lampsilini (Zanatta & Murphy, 2006: fig. 2). Although Bayesian inference (BI), posterior probability (PP) indicating amblemine affinities for *Popenaias* was quite high in Campbell et al. (2005: 0.99, fig. 2), the PP of the BI analysis in Zanatta & Murphy (2006: fig. 2) and maximum parsimony (MP) bootstrap percentages in both studies were less than 0.5 and 55, respectively. Originally described as *Unio popeii* Lea, 1857, previous classifications have been rather inconsistent regarding the phylogenetic propinquity of this species. Simpson (1900, 1914), Cockerell (1902), and Hinckley (1907) retained it within *Unio* Philipsson, 1788, but Pilsbry (1909a, b) listed it as a *Lampsilis* Rafinesque, 1820. Based on anatomical features including a presumed ectobranchus marsupium and similarities of anatomy, shell shape, and beak sculpture, Ortmann (1912) classified it under *Elliptio* Rafinesque, 1820. Frierson (1927) also classified it as an *Elliptio* but erected a new subgenus, *Popenaias*, with it as the type species and including two Mexican taxa. Heard & Guckert (1971) elevated *Popenaias* to generic status with two species (*popeii* and *buckleyi* Lea, 1843) and proposed Popenaiadinae for *Popenaias* and *Cyrtonaias*, based primarily on reproductive periodicity and "homogenae" marsupial morphology. However, Heard (1974) abandoned this subfamilial classification after determining that the diagnoses for Popenaiadinae were based on species specific characters not warranting higher classification. Johnson (1972, 1999) re-established *E. buckleyi* and reclassified *Popenaias* into Pleurobeminae Hannibal, 1912, respectively. Fuller (1975) established *Cyrtonaias* as a lampsiline. Subsequently, Smith et al. (2003) concluded that *P. popeii* had pleurobemine-like (Ambleminae: Pleurobemini) anatomy and, while typically exhibiting ectobranchy, demonstrated that *P. popeii* can brood embryos in the inner demibranchs facultatively, an anatomical condition distinct from the brooding condition in the above-mentioned genera. Given the above, the evolutionary relationships of both the Lampsilini and *P. popeii* remain inadequately understood.

Freshwater unionoidean bivalves, as well as representatives of the two marine bivalve orders Mytiloida and Veneroida, exhibit doubly uniparental inheritance (DUI) of mitochondrial DNA (mtDNA), which involves distinct maternal (F) and paternal (M) transmission routes concomitant with highly divergent gender-associated mtDNA genomes (Hoeh et al., 1996, 2002; Liu et al., 1996; Curole & Kocher, 2002, 2005; Walker et al., 2006; for a general review of DUI, see Breton et al., 2007). Females transmit their mitochondria (carrying F mtDNA) to sons and daughters, as in standard maternal inheritance, but males effectively transmit their mitochondria (via sperm carrying M mtDNA) only to sons (e.g., Sutherland et al., 1998; but see Obata et al., 2007; Chakrabarti et al., 2007). In males, F mtDNA predominates in the somatic tissues while principally M mtDNA is found in the testes. Thus, this genetic system yields homoplasmic (= containing a single mtDNA type) female and heteroplasmic (= containing multiple mtDNA types) male individuals. Intra- and inter-specific comparisons suggest that the M genome is evolving more rapidly than the F genome (Skibinski et al., 1994; Rawson & Hilbish, 1995; Stewart et al., 1995; Liu et al., 1996; Hoeh et al., 2002; Krebs, 2004). The F and M mitochondrial genomes of unionoidean bivalves form reciprocally monophyletic groups (Curole & Kocher, 2002, 2005; Hoeh et al., 1996, 2002; Walker et al., 2006) and are highly divergent (Mizi et al., 2005). Fossil evidence suggests that the F/M divergence, and concomitant independent evolution of F and M mitochondrial genomes, occurred > 200 MYA (Watters, 2001). Phylogenetic analyses of unionoidean species, using DNA sequences from both mtDNA lineages within a species as distinct terminals, typically yields well supported F and M clades with very similar topologies (Hoeh et al., 1996, 2002; Curole & Kocher, 2002, 2005; Krebs, 2004; Walker et al., 2006). Thus, concatenating F and M sequences (i.e., using F and M sequences additively to represent a single terminal) for phylogenetic analyses is justifiable, and the resulting trees are often more robustly supported (e.g., Hoeh et al., 2002; Walker et al., 2006) than those restricted to analyses of only F genome sequences (e.g., Campbell et al., 2005; Zanatta & Murphy, 2006).

The phylogenetic relationships of *Popenaias popeii* and other amblemine-like species near the ancestral lineage of the lampsiline clade remain unresolved (e.g., Campbell et al., 2005; Zanatta & Murphy, 2006). Inconsistencies of morphologically based classifications with the phylogenetic trees presented in Campbell et al. (2005) and Zanatta & Murphy (2006) further indicate the need for additional assessment of the evolutionary relationships of these taxa. To clarify these matters, matrices containing 2,310 nucleotide (nt) and 770 amino acid (a.a.) positions were constructed with sequences generated from five gene regions encoded on the F and M mitochondrial genomes of 21 amblemine species. Our phylogenetic analyses addressed phylogenetic relationships in the Ambleminae with special reference to the Lampsilini and pertinent evolutionary relationships indicated in the evolutionary trees of Campbell et al. (2005) and Zanatta & Murphy (2006).

MATERIALS AND METHODS

Taxa Used

We obtained sequences from a thorough cross-section (n = 21 species; Table 1) of the unionoidean bivalve subfamily Ambleminae, including 14 genera (16 species) representing the Amblemini and Lampsilini (*sensu* Campbell et al., 2005), two genera (two species) from the Pleurobemini, one genus (two species) representing the Quadrulini, and one species from the Gonideini. Collection locality information for the specimens utilized herein is presented in Appendix I. The use of *Inversidens japanensis* (Gonideini) as the outgroup in our phylogenetic analyses is justified by the results of Campbell et al. (2005) and Walker et al. (2006). Paleontological evidence suggests that the sequences analyzed herein diverged from a common ancestor > 65 MYA (Watters, 2001).

DNA Sequencing

Gender of each specimen was determined by microscopical examination of gonadal tissues. Total genomic DNA was isolated from mantle and testes using the Qiagen DNeasy animal kit. The largely M-specific primer pair from Chakrabarti et al. (2006) along with those in Walker et al. (2007) were used to amplify the M*cox2-cox1* junction region (Curole & Kocher, 2002) from testicular tissue-based DNA isolates. These primers amplified an

~1.7 kbp fragment from the M genomes. A largely F-specific primer pair (Walker et al., 2006) was used to amplify the corresponding F*cox2-cox1* junction region from mantle tissue-based DNA isolates. These primers amplified a ~1.1 kbp fragment from the F genome. The actual number of nucleotides used in the phylogenetic analyses (2,310 nt) is somewhat less that that suggested by the above fragment lengths due to the deletion of primer sequences, intergenic spacer regions and regions containing indels (see Results and Discussion).

TABLE 1. Species and GenBank accession numbers for the DNA sequences used in this study. Female-transmitted cytochrome *c* oxidase subunit I (F*cox1*); female-transmitted cytochrome *c* oxidase subunit II (F*cox2*); male-transmitted cytochrome *c* oxidase subunit I (M*cox1*); and male-transmitted cytochrome *c* oxidase subunit II (M*cox2*). Tribal assignments are as in Campbell et al. (2005: fig. 2 and text).

Species	Tribe	F*cox1*	F*cox2*	M*cox1*	M*cox2*
Inversidens japanensis (Lea, 1859)	Gonideini	AB055625	AB055625	AB055624	AB055624
Quadrula quadrula (Rafinesque, 1820)	Quadrulini	EF033268	EF033288	EF033308	EF033328
Quadrula refulgens (Lea, 1868)	Quadrulini	EF033269	AF517643	EF033309	AF517638
Fusconaia flava (Rafinesque, 1820)	Pleurobemini	EF033261	EF033281	EF033307	EF033327
Pleurobema sintoxia (Rafinesque, 1820)	Pleurobemini	EF033253	EF033273	EF033291	EF033311
Amblema plicata (Say, 1817)	Amblemini	EF033258	EF033278	EF033295	EF033315
Popenaias popeii (Lea, 1857)	Amblemini	EF033257	EF033277	EF033294	EF033314
Actinonaias ligamentina (Lamarck, 1819)	Lampsilini	EF033263	EF033283	EF033300	EF033320
Cyrtonaias tampicoensis (Lea, 1838)	Lampsilini	EF033259	EF033279	EF033299	EF033319
Glebula rotundata (Lamarck, 1819)	Lampsilini	EF033264	EF033284	EF033304	EF033324
Hamiota subangulata (Lea, 1840)	Lampsilini	EF033266	EF033286	EF033305	EF033325
Lampsilis hydiana (Lea, 1838)	Lampsilini	EF033270	------------	EF033298	EF033318
Lampsilis ovata (Say, 1817)	Lampsilini	EF033262	EF033282	EF033303	EF033323
Lampsilis straminea (Conrad, 1834)	Lampsilini	EF033271	EF033289	EF033297	EF033317
Lemiox rimosus (Rafinesque, 1831)	Lampsilini	EF033256	EF033276	EF033302	EF033322
Obliquaria reflexa (Rafinesque, 1820)	Lampsilini	EF033254	EF033274	EF033292	EF033312
Obovaria olivaria (Rafinesque, 1820)	Lampsilini	EF033267	EF033287	EF033306	EF033326
Plectomerus dombeyanus (Valenciennes, 1827)	Lampsilini	EF033252	EF033272	EF033290	EF033310
Ptychobranchus fasciolaris (Rafinesque, 1820)	Lampsilini	EF033265	EF033285	EF033301	EF033321
Toxolasma lividus (Rafinesque, 1831)	Lampsilini	EF033255	EF033275	EF033293	EF033313
Venustaconcha ellipsiformis (Conrad, 1836)	Lampsilini	EF033260	EF033280	EF033296	EF033316

PCR reactions consisted of 1X Qiagen PCR buffer, 0.2 mM each dNTP, 0.5 M each primer, 1U Qiagen *Taq* and ~20 ng of template DNA. Reactions using the M-specific primers were cycled at 94°C for 60 s, 50°C for 60 s, and 72°C for 120 s for a total of 40 cycles. Reactions involving the F-specific primers followed the same profile as above, but were annealed at 46°C. The above PCR primers ultimately yielded F and M*cox2-cox1* DNA sequences obtained via cycle sequencing with Perkin Elmer AmpliCycle Sequencing Kits. Sequencing primers were identical in sequence to the PCR primers and sequencing template purification was done following Folmer et al. (1994). Sequences were visualized using Li-Cor 4200L-2 and 4200S-2 DNA sequencers. Forward and reverse sequencing reads were assembled and verified using AlignIR (version 2.0, LI-COR, Inc.), and final sequence alignments were completed manually with MacClade version 4.0 (Maddison & Maddison, 2000).

Phylogenetic Analyses

Our M*cox2* sequences were aligned with unionoidean F*cox2* nucleotide sequences (which have a uniform length) obtained from the GenBank and herein to determine the boundaries and length of M*cox2*e, the hypervariable coding extension of the M*cox2* gene specific to unionoid M mtDNA (Curole & Kocher, 2002; Walker et al., 2007). The 5' end of M*cox2*e is designated as the nucleotide of the M*cox2* sequence that aligns with position one in the stop codon of the F*cox2* sequences. The 3' end of M*cox2*e is the stop codon for M*cox2*. The F and M*cox2-cox1* nucleotide sequences were translated to protein sequences using the *Drosophila* mtDNA genetic code. All DNA sequences generated herein were submitted to the GenBank database (Table 1).

Phylogenetic trees were estimated using Bayesian inference (BI), neighbor-joining (NJ) and maximum parsimony (MP) approaches. Bayesian analyses were conducted using the program MrBayes (version 3.1.2: Huelsenbeck & Ronquist, 2005; Ronquist & Huelsenbeck, 2003). For nucleotide sequences, Bayesian searches were run for 10 million generations with 10 search chains, and the data were partitioned by gene region and codon position (five gene regions × three codon positions for the full data-set; four gene regions × three codon positions for the data-set with the M*cox2*e sequences removed), saving 10,000 trees (one tree saved every 1,000 generations) and using GTR + G + I substitution model (Rodriguez et al., 1990) as selected by the program Modeltest (Posada & Crandall, 1998). To allow each partition to have its own set of parameter estimates, *revmat*, *tratio*, *statefreq*, *shape*, and *pinvar* were all unlinked during the analysis. Burn-in was determined by visual inspection of the likelihood score plots obtained as the trees were written to the tree file. In all analyses, stationarity was reached before one million generations, and the first 1,000 trees were discarded (i.e., the first million generations) from each analysis as the burn-in. To obtain the most accurate branch length estimates possible, the option *prset ratepr = variable* was employed per the recommendations of Marshall et al. (2006). To evaluate the effects that a simpler substitution model could have on tree topology, we also conducted analyses using the HKY + G model (Hasegawa et al., 1985).

We analyzed protein sequences translated from the concatenated 2,310 nt data-set (total of 770 a.a., including 143 unambiguously aligned M*cox2*e a.a.). Finally, we analyzed protein sequences translated from the concatenated 1,881 nt data-set (total of 627 a.a.) without M*cox2*e, again to determine whether the inclusion of the extension sequences had any effect on the overall topology. For protein data-sets, Bayesian searches were run for five million generations with eight search chains, saving 10,000 trees, and using the Mtrev [*prset aamodelpr = fixed(mtrev)*] substitution model (Adachi & Hasegawa, 1996a, b) with the data partitioned by gene region. The analyses of both protein sequence data-sets reached stationarity before 500,000 generations, and the first 1,000 trees were discarded as burn-in. Thus, for both nucleotide and protein data-sets, the Bayesian analyses were run for at least 10 times as long as each took to reach stationarity. Reliability of the Bayesian topologies was evaluated with the posterior probabilities from the majority-rule consensus trees.

Neighbor-joining analyses of the nucleotide and protein data-sets were done using *MEGA3* (Kumar et al., 2004). Reliability of the internal nodes of the NJ trees was estimated by bootstrapping the data-set with 1,000 replications. A branch-and-bound maximum parsimony search was conducted on the concatenated 2,310 nt data-set (which was transformed at only the 3rd codon positions [wherein only transversions were coded]) using PAUP* (version 4.0β10: Swofford, 2001). A total of 100,000 full heuristic MP bootstrap replications was done to estimate the reliability of the internal nodes.

Differences in topology between constrained BI trees (based on the topologies in Campbell et al., 2005, and Zanatta & Murphy, 2006, as well as from hypotheses of evolutionary relationships deduced from classifications of Hannibal, 1912, and Heard & Guckert, 1971) and the best BI tree from the unconstrained BI analysis of the complete nucleotide data-set were tested with the likelihood-based approximately unbiased test (AU: Shimodaira, 2002), the Shimodaira-Hasegawa test (SH: Shimodaira & Hasegawa, 1999), the weighted Shimodaira-Hasegawa test (WSH: Shimodaira, 2002), the Kishino-Hasegawa test (KH: Kishino & Hasegawa, 1989), and the weighted Kishino-Hasegawa test (WKH) in *CONSEL* (Shimodaira & Hasegawa, 2001). The program *Mesquite* (version 1.05: Maddison & Maddison, 2003) was used to implement maximum likelihood estimations of ancestral character states on the best BI tree estimated from the 2,310 bp data-set under the GTR + G + I model. Both the "Markov k-state 1 parameter model" (MK1: Lewis, 2001), which assumes equally probable forward and backward rates of change, and the "Asymmetrical Markov k-state 2 parameter model" (AsymmMk: Pagel, 1997; Mooers & Schluter, 1999), in which "forward" and "backward" transition rates can be different, were investigated. The asymmetry likelihood ratio test was used to determine whether the AsymmMK model was significantly better than the MK1 model (see the *Mesquite* manual). To assess which ancestral character state was best for a given node, state estimates with a log likelihood 2 or more units lower than the best state estimate (decision threshold [T] set to T = 2) were rejected (Edwards, 1972; Pagel, 1999). *Mesquite* also was used to produce MP-based estimates of ancestral character states using the best BI tree topology.

RESULTS AND DISCUSSION

In the 21 species examined, we obtained comparable sequences of the following lengths: 672 bp of F*cox1*, 279 bp of F*cox2*, 651 bp of M*cox1*, and 279 bp of M*cox2*h. M*cox2e* ranged from 543 (*Inversidens* and *Amblema*) to 561 (*Ptychobranchus*) bp in length (181–187 a.a.). The M*cox2*e region contains indels within nucleotide positions 7–135. The first six nucleotides and those from position 136–558 aligned unambiguously and were used in analyses containing M*cox2*e, while those from positions 7–135 were removed prior to analyses.

The best tree from the Bayesian analysis of the concatenated M and F*cox2-cox1* nucleotide sequences (including M*cox2*e) using GTR + G + I is shown in Figure 1. Therein, the general amblemine tribal relationships indicated in Campbell et al. (2005) – e.g., (Quadrulini (Pleurobemini (Amblemini, Lampsilini))) – are supported but with some notable exceptions near the ancestral lineage of the Lampsilini. In Figure 1, *Popenaias popeii* is represented as the sister taxon to a clade composed of all of the lampsiline species included in this data-set. This grouping is supported by a Bayesian inference posterior probability (BI PP) of 1.00 and a MP bootstrap percentage (MP BSP) of 100. The basal position of *P. popeii* relative to the lampsiline species included herein is supported by a BI PP of 1.00 and a MP BSP of 94 (Fig. 1). Given these high nodal support values, this is the first robust demonstration of the monophyly of the Lampsilini; both Campbell et al. (2005) and Zanatta & Murphy (2006) had BI PPs < 0.95 and MP BSPs < 50. Furthermore, all of the variations on our Bayesian analyses (including and omitting M*cox2*e; nt vs. a.a. data) had BI PPs ≥ 0.97 for the monophyly of the Lampsilini (Appendix II). The inclusion of *Amblema* and *Plectomerus* in a clade with the traditional lampsiline taxa + *Popenaias* is strongly supported (BI PP = 1.00, MP BSP = 96), while their basal placement to the traditional lampsiline taxa + *Popenaias* is supported by a BI PP of 1.00 and a MP BSP of 100 (Fig. 1). Therefore, the hypothesis that *Plectomerus* and *Toxolasma* are sister genera (Campbell et al., 2005: fig. 2) is strongly rejected by these results. The paraphyletic nature of *Amblema* + *Plectomerus* is also supported by the present analysis (PP = 0.98, MP BSP = 73). The *Lampsilis*-like species represented in this data-set (i.e., *L. ovata*, *L. hydiana*, *L. straminea*, *Actinonaias ligamentina*, and *Hamiota subangulata*) comprise a very well-supported (BI PP = 1.00, MP BSP = 100), relatively derived clade in the best BI tree (Fig. 1).

The Bayesian analysis of the five gene region data-set using the HKY + G model yielded an identical topology. The Bayesian analysis, using GTR + I + G, that omitted the M*cox2*e region was essentially the same, the only dif-

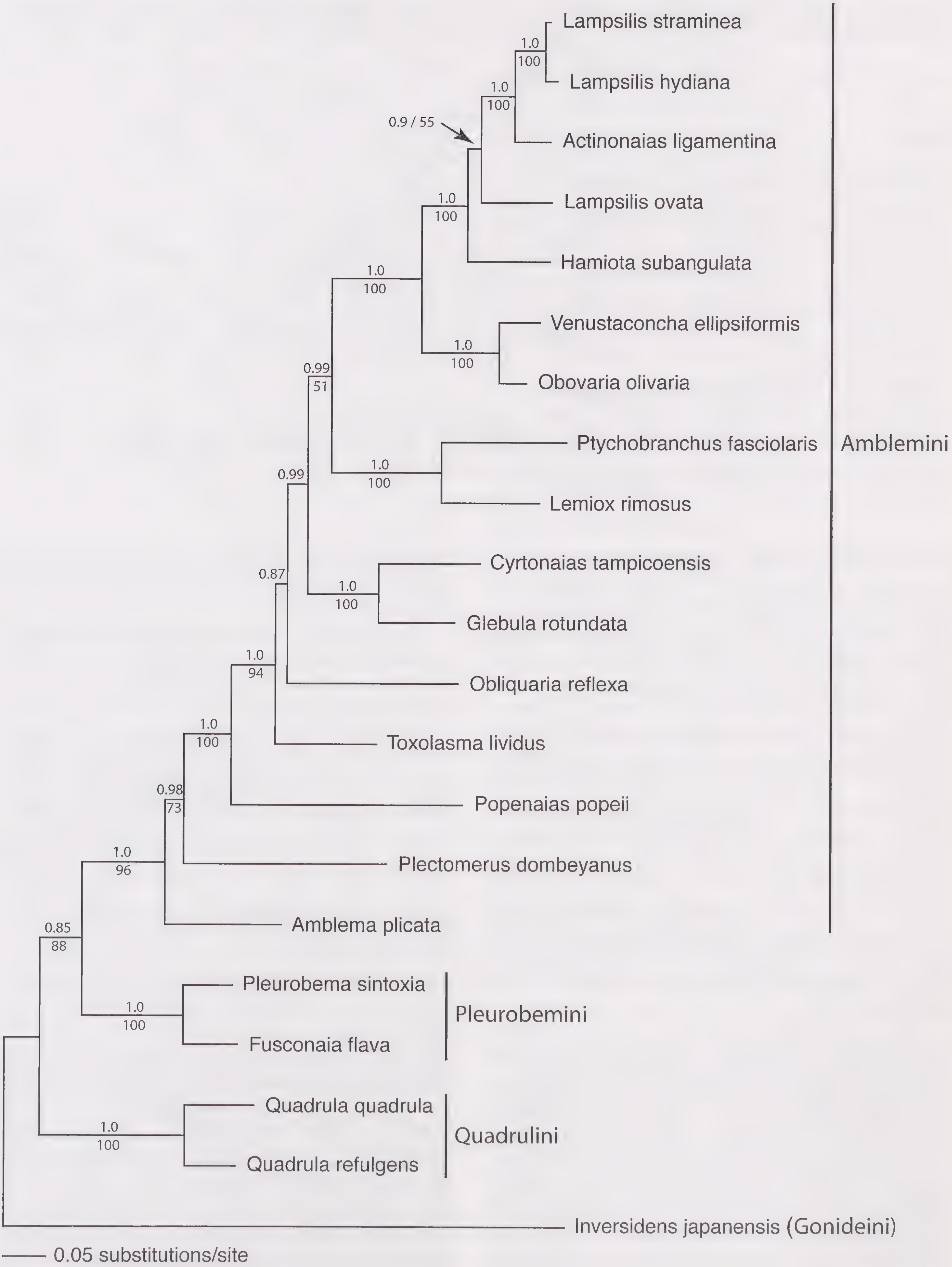

FIG. 1. Bayesian tree, for the 21 amblemine bivalve species, with highest overall posterior probability. This tree was generated using the GTR+I+G model on sequences from five mitochondrial gene regions, displays maximum likelihood estimated branch lengths and shows nodal support values (PP above branch; MP bootstrap percentage below branch [when > 50%]).

TABLE 2. BI constraint analysis results using the five gene region, nucleotide dataset. Only two (in parentheses) of the thirty "best tree vs. constraint tree" comparisons were deemed insignificant.

			Likelihood-based tests				
Tree	-ln L	Difference	AU	KH	SH	WKH	WSH
Unconstrained	19408.96	(Best)					
Constraint 1	19477.30	68.34	p = 2e-06	p = 1e-04	p = 2e-04	p = 1e-04	p = 1e-04
Constraint 2	19431.05	22.10	p = 0.014	p = 0. 019	(p = 0.123)	p = 0.019	p = 0.038
Constraint 3	19431.05	22.10	p = 0.014	p = 0. 019	(p = 0.123)	p = 0.019	p = 0.038
Constraint 4	19495.67	86.71	p = 1e-91	p = 0	p = 0	p = 0	p = 5e-05
Constraint 5	19460.96	52.00	p = 2e-04	p = 0.001	p = 0.001	p = 0.001	p = 0.001
Constraint 6	19488.49	79.54	p = 1e-04	p = 4e-05	p = 4e-05	p = 4e-05	p = 4e-05

Constraint 1 = (*Plectomerus* + *Toxolasma*)
Constraint 2 = (*Popenaias* + *Amblema*)
Constraint 3 = (*Plectomerus*, ((*Popenaias* + *Amblema*), all lampsilines))
Constraint 4 = ((*Popenaias*, *Amblema*), ((*Plectomerus*, *Toxolasma*), all other lampsilines))
Constraint 5 = (lampsilines + *Popenaias* + pleurobemines)
Constraint 6 = (lampsilines + pleurobemines)

ference being a three-clade polytomy near the root that was resolved in Figure 1 (Appendix II-A). The analysis of the data-set omitting M*cox2*e using HKY + G had *Amblema* and *Plectomerus* exchanging positions, and a clade comprised of the two *Quadrula* species was sister to *Pleurobema* + *Fusconaia* (Appendix II-B). Nevertheless, the evolutionary relationships indicated by our tree topology in Figure 1 are generally similar to those in Campbell et al. (2005) and Zanatta & Murphy (2006); however, our Figure 1 typically displays higher nodal support values. This could be due simply to a larger number of informative characters in our most inclusive nucleotide matrix (914 parsimony-informative characters; Campbell et al., 2005: 749 parsimony-informative characters; Zanatta & Murphy, 2006: 606 parsimony-informative characters).

The topology from the BI analysis of the protein sequences (Mtrev substitution model) including M*cox2*e had only minor differences from the tree in Figure 1; *Hamiota* + *Lampsilis ovata* and (*Cyrtonaias* + *Glebula*) + (*Ptychobranchus* + *Lemiox*) were paired as sister taxa with both pairings exhibiting low nodal support (BI PP = 52 and 66, respectively; Appendix II-C). The topology from the BI analysis with M*cox2*e protein sequences deleted had the following differences from the topology in Figure 1: *Actinonaias* + *Hamiota* were paired as sister taxa with *Lampsilis ovata* sister to (*Hamiota* + *Actinonaias*) + (*L. straminea* + *L. hydiana*), a clade comprised of the two *Quadrula* species were sister to *Pleurobema* + *Fusconaia* and *Plectomerus* was sister to a clade that included all taxa except *Inversidens* (Appendix II-D).

The neighbor-joining analyses produced essentially the same tree topologies as Bayesian methods, although the bootstrap support for some internal nodes was quite low (Appendices III, IV). The branch-and-bound maximum parsimony search on the transformed nucleotide matrix yielded two most parsimonious trees (not shown) highly similar to the topology in Figure 1; however, in one, *Toxolasma lividus* and *Obliquaria reflexa* were paired as sister taxa, while the other tree returned *Obliquaria* as sister to ((*Lemiox* + *Ptychobranchus*) + (*Obovaria* + *Venustaconcha*) + (*Hamiota* + (*L. ovata* + (*Actinonaias* + (*L. straminea*+ *L. hydiana*))))). Of our 11 explicitly figured trees, ten displayed *Popenaias* as the sister taxon to a clade containing the traditional lampsiline taxa. The exception is a NJ tree where *Popenaias* is sister to *Amblema* but with a NJ BSP of < 50 (Appendix III-D).

With respect to *Amblema*, *Plectomerus*, *Popenaias*, and *Toxolasma*, results of the constraint analyses (Table 2) strongly suggest that the evolutionary relationships implied by our best BI tree (Fig. 1) are significantly better than those presented in Campbell et al. (2005: figs. 1, 2) and Zanatta & Murphy (2006: fig. 1). Specifically, constraint statements #1 (*Plectomerus* + *Toxolasma*) and #4 ((*Popenaias*, *Amblema*), ((*Plectomerus*, *Toxolasma*), all other lampsilines)) (Campbell et al., 2005: fig. 2) produced trees that were significantly worse than the

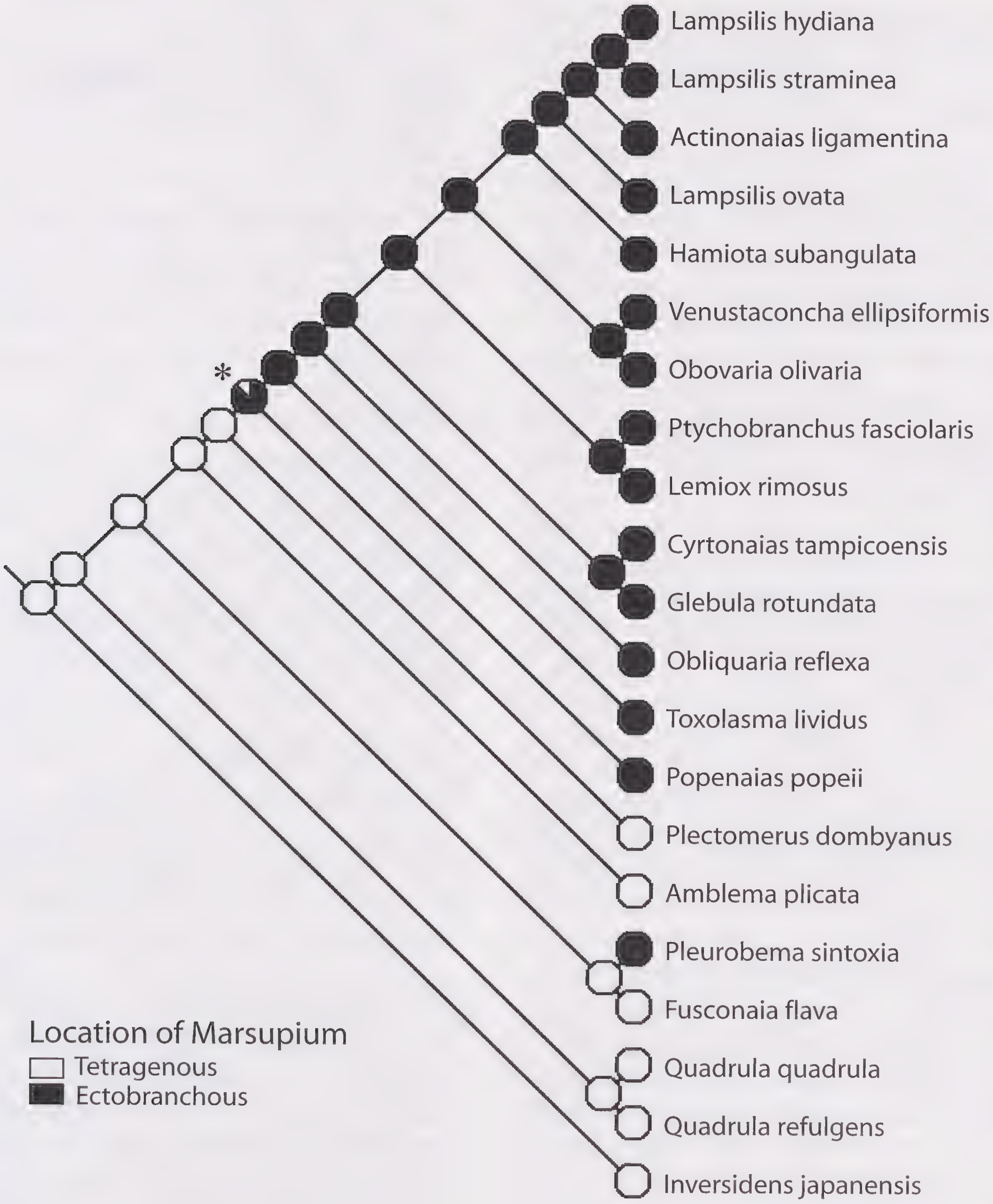

FIG. 2. ML ancestral character state estimation of the location of the marsupium, for the 21 amblemine bivalve species, using the Asymmetrical Markov k-state 2 parameter model on the tree presented in Figure 1. The asterisk (*) denotes the only node that is not significant for the state occupying the majority of each pie chart.

unconstrained tree (Fig. 1) by all five ML test statistics (Table 2). Similarly, constraint statements #2 (*Popenaias* + *Amblema*) and #3 (*Plectomerus*, ((*Popenaias*, *Amblema*), all lampsilines)) (Campbell et al., 2005: figs. 1, 2; Zanatta & Murphy, 2006: fig. 1) produced trees that were deemed significantly worse than the unconstrained tree (Fig. 1) by four out of the five ML test statistics (Table 2). Constraint statements #5 and #6, which evaluated the hypotheses that lampsilines have pleurobemine affinities (e.g., Hannibal, 1912; Heard & Guckert, 1971), also produced significantly worse topologies than the best unconstrained tree (Fig. 1).

Figure 1 is demonstrably the best current estimate of phylogeny for the present taxa/data combination. Using this tree, ML ancestral character state estimation procedures generated inferences regarding evolutionary transitions in the location of the marsupium for the included species (Fig. 2). For this character state optimization, the AsymmMK model was significantly better than the MK1 model and the former was used to optimize the location of the marsupium onto the tree in Figure 1. The ancestral character state estimations for all of the internal nodes of the tree were significant for the majority state present in each pie chart except for the node leading to

Popenaias (denoted by an asterisk). The MP estimate (not shown) was essentially identical to that from ML. Figure 2 indicates that the ectobranchy present in the "traditional" lampsiline taxa + *Popenaias* was derived a single time from the tetragenous brooding condition found in an *Amblema*/*Plectomerus*-like ancestor rather than from the plesiomorphic retention of pleurobemine ectobranchy. It is readily apparent that the evolution of ectobranchy was a necessary antecedent to the attainment of the specialized "heterogenae" brooding condition (i.e., posteriorly located marsupia in only the outer demibranchs) now found in the more derived lampsiline taxa.

The presence of a principally ectobranchous brooding location in *Popenaias*, the topologies presented in Figure 1 and Appendices II–IV and the constraint analysis results (Table 2) suggest that *Popenaias* has greater propinquity to the "traditional" lampsiline genera than to *Amblema* or *Plectomerus*. The phylogenetic placement of *Popenaias popeii* in Figure 1 and the occasional use of its inner demibranchs for brooding suggest that this species likely possesses some characteristics of the lampsiline ancestral lineage. Thus, further studies of *Popenaias*, *Amblema*, and *Plectomerus* morphology, reproduction, ecology and phylogeography combined with that of other pertinent taxa – e.g., "*Fusconaia*" *ebena* (Lea 1831), "*Obovaria" rotulata* (Wright 1899), *Actinonaias sapotalensis* (Lea 1841), *Friersonia iridella* (Pilsbry & Frierson 1908), and *Nephronaias*, *s.s.*, spp. (Ortmann, 1912; Simpson, 1914; Fuller, 1975; Campbell et al., 2005) – are essential to form a better understanding of the evolutionary antecedents to the extraordinary morphological radiation that occurred during lampsiline phylogenesis.

As mentioned in the Introduction, a narrow use of only the morphological "lampsiline diagnostic" character states would likely result in the exclusion of some species typically perceived to be "lampsiline." However, recent phylogenetic analyses utilizing molecular data have supported the monophyly of the traditional lampsiline species (e.g., Campbell et al., 2005; Zanatta & Murphy, 2006; trees presented herein), which strongly attests to the reality of the group. Given this situation and the lack of obvious patristic distance-based "gaps" near the base of the lampsiline/*Popenaias*/*Plectomerus*/*Amblema* clade in Figure 1, delimiting "What is a lampsiline?" herein would constitute a relatively subjective decision. Instead, we suggest the use of the tribal designation Amblemini for the lampsiline/*Popenaias*/*Plectomerus*/*Amblema* clade in Figure 1 because the usage of Amblemini Rafinesque, 1820, in a tribal context has priority over Lampsilini Ihering, 1901. This strategy eliminates the production of a paraphyletic Amblemini group, which would likely contain at least *Plectomerus* and *Amblema*, if a restricted lampsiline clade within the more inclusive group was designated as the Lampsilini. Furthermore, this implementation does not preclude a future recognition of a distinct lampsiline clade at the subtribal rank (i.e., Lampsilina).

Due to issues involving limited taxon sampling, it is readily apparent that the evolutionary relationships presented in this study are not conclusive regarding amblemine evolutionary relationships. However, the general agreement of our amblemine relationships, based on total molecular evidence analyses of sequences from two independent mtDNA genomes with those depicted in more taxonomically inclusive, F mtDNA genome-only studies (e.g., Campbell et al., 2005; Zanatta & Murphy, 2006) suggests that the broad outline of amblemine phylogeny has emerged. Furthermore, it should be noted that most of the parsimony-informative characters in the analyses presented herein came from the M mtDNA sequences, further illuminating the potential importance of M genomes in freshwater mussel phylogenetics (Hoeh et al., 2002; Walker et al., 2006). Subsequent studies of unionoidean bivalve evolution will benefit greatly from combined analyses of F mtDNA-, M mtDNA- and nucleus-encoded DNA sequences as well as the inclusion of morphological and behavioral characters.

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APPENDIX I

Collection locality information for the amblemine bivalve specimens sequenced herein. (Species authors and dates given in Table 1.)

Quadrula quadrula – East Fork of the White River, downstream of US 50/SR 37 bridge, Lawrence County, Indiana.

Quadrula refulgens – Amite River, near Port Vincent, East Baton Rouge Parish, Louisiana.

Fusconaia flava – Ohio River @ River Mile 625, near Fishtown, Harrison County, Indiana.

Pleurobema sintoxia – East Fork of the White River, downstream of US 50/SR 37 bridge, Lawrence County, Indiana.

Actinonaias ligamentina – French Creek @ Gravel Run Road crossing, Venango, Crawford County, Pennsylvania.

Amblema plicata – Black River @ Aitken Road crossing, Sanilac County, Michigan.

Cyrtonaias tampicoensis – Guadalupe River, upstream of Wood Lake and upstream of the access road from the town of Cost, Gonzales County, Texas.

Glebula rotundata – Bayou Carron at State Highway 10 crossing, near town of Washington, St. Landry Parish, Louisiana.

Hamiota subangulata – Tributary of Lower Flint River (Spring Creek) @ US 84 crossing, Decatur County, Georgia.

Lampsilis hydiana – Cossatot River @ State Highway 24 crossing, Sevier County, Arkansas.

Lampsilis ovata – Ohio River @ River Mile 726, Hancock County, Kentucky.

Lampsilis straminea – Brush Creek (Tombigbee River tributary) @ State Highway 14 crossing, Greene County, Alabama.

Lemiox rimosus – Duck River @ River Mile 179.1, Milltown, Marshall County, Tennessee.

Obliquaria reflexa – Duck River @ River Mile 179.1, Milltown, Marshall County, Tennessee.

Obovaria olivaria – St. Croix River at Interstate Park, near Taylors Falls, Chisago County, Minnesota/Polk County, Wisconsin.

Plectomerus dombeyanus – Ouachita R. @ Highway 79B crossing, Camden, Ouachita County, Arkansas.

Popenaias popeii – Black River (Pecos River drainage), Eddy County, New Mexico.

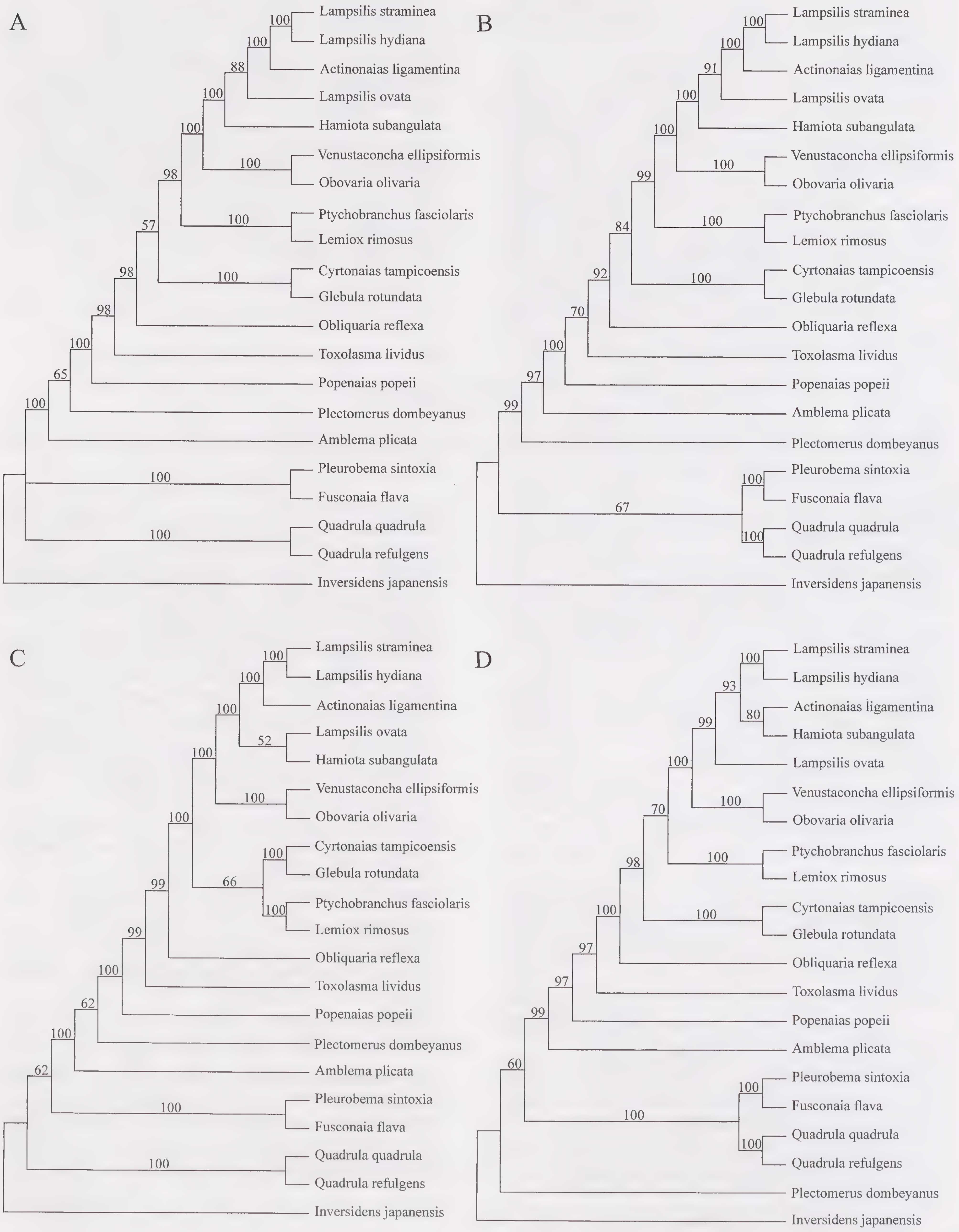
Ptychobranchus fasciolaris – French Creek @ Gravel Run Road bridge, Venango, Crawford County, Pennsylvania.

Toxolasma lividus – Clinton River, outflow of Dawsons Mill Pond, Beaudette Park, upstream of Orchard Lake Road crossing, Pontiac, Oakland County, Michigan.

Venustaconcha ellipsiformis – Sugar River, upstream of state Highway M-30 crossing, Gladwin County, Michigan.

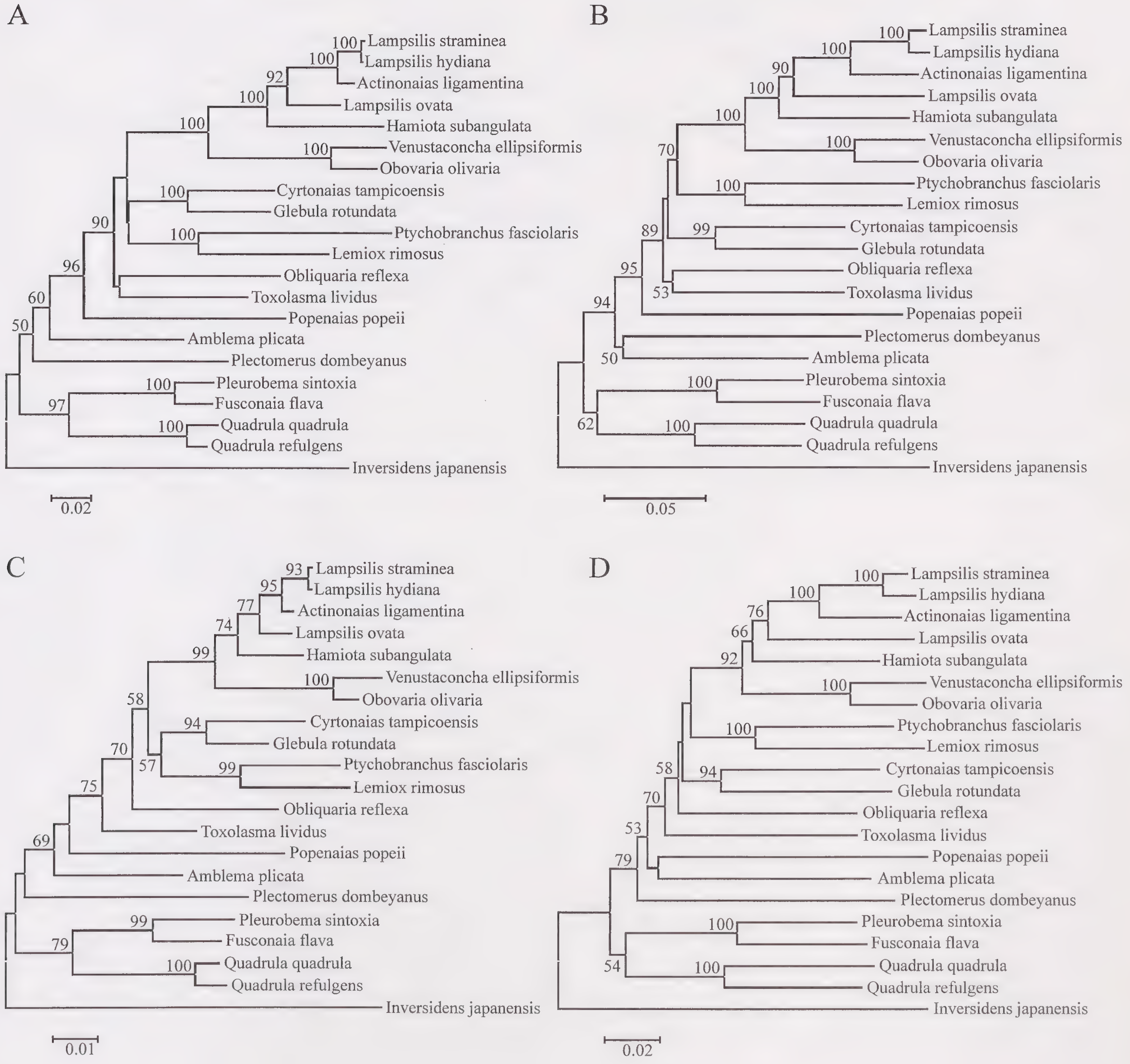
APPENDIX II

Consensus trees of 21 amblemine bivalve taxa from Bayesian analyses of (A) the nucleotide dataset excluding the M*cox2* extension using the GTR+G+I model; (B) the nucleotide dataset excluding the M*cox2* extension using the HKY+G model; (C) the amino acid dataset including the extension using the Mtrev model; (D) the amino acid dataset excluding the extension using the Mtrev model. Numbers above the branches are posterior probabilities x 100.



APPENDIX III

Neighbor-joining phylogenetic trees of 21 amblemine bivalve taxa. Bootstrap values are based on 1,000 replications, and only those greater than 50% are shown. *Inversidens japanensis* was used as outgroup. (A): Analysis based on concatenated partial amino acid sequences of M*cox1*, M*cox2*, F*cox1*, F*cox2* and M*cox2*e. Dayhoff's distance and complete-deletion option were used (628 shared sites); (B): Analysis based on concatenated partial nucleotide sequences of M*cox1*, M*cox2*, F*cox1*, F*cox2* and M*cox2*e. Tamura-Nei distance and complete-deletion option were used (1895 shared sites); (C): Analysis based on concatenated partial amino acid sequences of M*cox1*, M*cox2*, F*cox1*, F*cox2* (without M*cox2*e). Dayhoff's distance and complete-deletion option were used (485 shared sites); (D): Analysis based on concatenated partial nucleotide sequences of M*cox1*, M*cox2*, F*cox1*, F*cox2* (without M*cox2*e). Tamura-Nei distance and complete-deletion option were used (1,466 shared sites).



APPENDIX IV

Neighbor-joining phylogenetic trees of 21 amblemine bivalve taxa. Bootstrap values are based on 1,000 replications, and only those greater than 50% are shown. *Inversidens japanensis* was used as outgroup. (A): Analysis based on partial amino acid M*cox2*e sequences. Dayhoff's distance and complete-deletion option were used (143 shared sites); (B): Analysis based on M*cox2*e nucleotide sequences. Tamura-Nei distance and complete-deletion option were used (429 shared sites). Dayhoff's distance, computed as Poisson-corrected gamma distance with a = 2.25 (Nei & Kumar, 2000), was used to take into account parallel and backward substitutions.